Dynamic Camouflage in Benthic and Pelagic Cephalopods: An Interdisciplinary Approach to Crypsis Based on Color, Reflection, and Bioluminescence

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LONG-TERM GOALS

Our overall goal is to understand the perceptual and mechanistic principles that underlay camouflage framed in the context of the animals' environment. In particular, we plan to characterize and understand the perceptual abilities of several species of benthic and pelagic cephalopods (which are unrivaled masters of dynamic camouflage), the aspects of their optical environment that affect their camouflage behavior, the characterization of that behavior, and the molecular mechanisms inside the skin by which those responses are accomplished.

OBJECTIVES

1. To fully characterize the spatiotemporal characteristics of the near-surface and shallow benthic underwater light field, including ultraviolet radiation and polarization.

2. To determine the visual abilities of several species of cephalopod and model both the shallow and deep-water world from the animals' points of view.

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3. To incorporate the knowledge gained from tasks 1 and 2 in order to study the camouflage behavior of these species under simulated ocean conditions. This will lead to an understanding of the most important aspects of their environment that determine their optical camouflage response.

4. To understand the underlying molecular and biophysical mechanisms governing changes in the skin that produce the observed optical effects, to help provide a platform for future translational efforts.

APPROACH

Figure 1: Core study species. Left: Pterygioteuthis microlampas a) animal in white light b) counterillumination. Middle: Loligo opalescens. Right: Octopus bimaculoides

Our core study species are the following:

1. ***Pterygioteuthis microlampas*:** a small counterilluminating squid that changes the color of its emitted light via reflectin-based tunable interference filters.

2. ***Loligo opalescens*:** a moderately-sized near-surface squid with dynamic iridophores and chromatophores that is our molecular team's current model system for understanding reflectin self-assembly.

3. ***Octopus bimaculoides*:** a small, hardy benthic octopus with base-layer iridophores, chromatophores and two rings of intense blue iridescence.

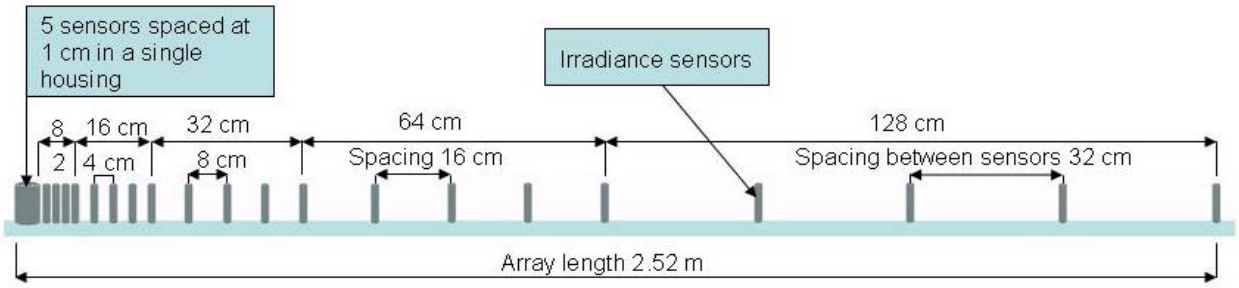

Figure 2: The proposed Spatial Radiometric Array (SRA).

Objective 1 will be achieved via the development and use of two novel optical instruments: 1) the Underwater Porcupine Radiometer System and 2) the Spatial Radiometric Array (SRA). The

Porcupine (developed during ONR's RaDyO program) uses 23 sensors to measure downwelling spectral irradiance and radiance at 532 nm in multiple directions at a sampling rate of 1 kHz. The SRA, which will be developed during this project, is a linear array of 25 downwelling irradiance sensors designed to acquire one-dimensional spatial statistics (@ 532 nm). The orientation of the sensor array can be positioned to acquire spatial statistics along any desired direction (e.g. predominant wind/wave direction, solar azimuthal plane). The photodetectors and data acquisition system of the SRA will be similar to those on the Porcupine, which are designed to meet the requirement of simultaneous sampling with multiple detectors at the very high rate of 1 kHz. However, the physical dimensions of the SRA's sensors will be smaller, allowing us to resolve spatial scales of fluctuations down to ~1 cm. The first sensor to the left in that housing serves as the reference sensor for calculations of the spatial autocorrelation function. The remaining sensors of the array are equally spaced at 2, 4, 8, 16, and 32 cm within sections in such a manner that there are five sensors per section. Both the SRA and Porcupine instruments will be deployed on the sea bottom during *in situ* observations of animals at the Catalina field station. The SRA will provide both spatial and temporal statistics primarily at a single "standard" wavelength of 532 nm. The Porcupine will collect temporal statistics of E_d at 7-10 different wavelengths from the near UV (365 nm) to the red (670 nm), and temporal statistics of L_d at 13-16 zenith directions within two orthogonal azimuthal planes at 532 nm. As a result, using these two instruments with nearly 50 photodetectors, we will obtain unique data sets that will characterize fluctuations in spectral irradiance both in terms of temporal and spatial statistics in conjunction with data on radiance fluctuations. We will acquire time-series at several depths and under various environmental conditions (wind/waves, sun/sky illumination, solar angle, and water optical properties). Similar benthic experiments are proposed for the optional period in the tropical reef environment at Palau where additional animal species will be studied. The Porcupine instrument will also be used to measure temporal fluctuations in the pelagic environment during the two cruises. In addition to the SRA and Porcupine, we will also use commercial hyperspectral radiometers (Satlantic, Inc., Biospherical PUV-500 for UV) to characterize downwelling and upwelling light fields during all experiments. These commercial instruments will provide time-averaged data (averaging time > 0.5 - 1 s) with high spectral and vertical resolution throughout the water column.

Objective 2 involves measuring six primary visual parameters of the study species: field of view, spectral sensitivity, acuity, temporal resolution, and contrast and polarization sensitivity. Field of view is determined from the placement and orientation of the eyes and the geometry of the retina and pupil. Spectral sensitivity will be investigated using microspectrophotometry (MSP), which measures the absorption spectra of individual photoreceptors. Acuity will be estimated from the spacing of photoreceptors in the retina and via a robust assay known as the optomotor response, in which an animal's eyes or body follow a large moving stimulus. Using a striped cylinder of increasingly finer stripes, one can determine the acuity. By changing the speed of the rotation, one can also assay the temporal resolution. Contrast sensitivity is usually determined by measuring the contrast threshold C_{min}, the smallest detectible fractional difference in intensity. This will be estimated by determining photon catch and also via optomotor assays using stripes of decreasing contrast. Polarization sensitivity will also be assayed via retinal morphology and an optomotor response to a cylinder whose stripes differ only in e-vector orientation. The information about the visual parameters will be used to model the visual world using standard Fourier and other image processing techniques.

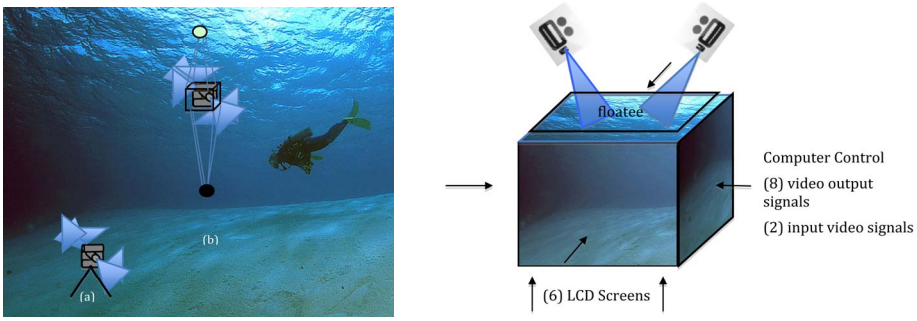

Figure 3: Left) Janus camera system. Right) holodeck.

Camouflage behavior (objective 3) will be studied inside a "holodeck", a tank surrounded by monitors that project natural environments or controlled visual stimuli. The top of the tank will have a plexiglass "floatee" that will make the surface optically flat and permit undistorted observation of the animals from the outside as well as permitting images to be projected into the tank by two DLP projection systems. The tank will be built and tested at SIO in the experimental aquarium facility and then transported to UCSB where it will be set up in a lab equipped with a flow-through filtered and thermostated sea water system. Animals will be placed inside the tank and after acclimatization subjected to different visual environments. Movies of natural environments will come from another novel device that we call the "Janus Camera": a collection of six underwater video cameras that image the environment in all six axes. Alternatively, synthetic images can be displayed. The synthetic and natural images will be used to answer the following three major questions:

1. how much of cephalopod camouflage is passive?

2. what aspects of a scene does a cephalopod use to determine camouflage?

3. in what ways do the spatiotemporal statistics of the dynamic reflectance of a cephalopod correlate with the spatiotemporal aspects of the light field?

In general the holodeck will be used in the following way. Animals will be exposed to different visual environments and videotaped. Control sessions, in which gray cards and models are substituted for the animal, will be used to measure changes in illumination, that can be combined with the changing radiance of the animals to determine the changing reflectance of their skin. These time series of changing reflectance will then be analyzed using Fourier and other spectral methods to determine their statistical properties in time and space (and in both achromatic and chromatic channels). These will be compared with various statistical properties of the overhead illumination and different portions of the scene depending on the needs of the particular experiment.

The fourth objective is to quantify the correspondence between the optical properties of cephalopod skin and their optical environments and to uncover the biophysical principles driving the neurotransmitter-induced self-assembly of the photonically active reflectin proteins. Our preliminary observations show that *Octopus bimaculoides* has a greenish, unpolarized reflectance in its murky, greenish light environment, while *Loligo opalescens*, living in the optically dynamic pelagic region has polarized iridescence that is changeable in both brightness and color. The measurements described here will allow us to precisely quantify this observed match between these reflective skin surfaces and the optical parameters of the water column. We will characterize these parameters using fiber-optic

spectroscopy coupled with goniometry, measuring the polarization-specific bidirectional reflectance of the skin of the target species and correlate these with the statistical analyses of the light measurements from task 1 to determine which aspects of this complex reflectance have specifically evolved for camouflage. We will also determine the ultrastructure of the reflectin-based structures using transmission electron microscopy and model their optical effects to determine what aspects of the biological structures are important for the observed environmental optical match.

The dynamic iridescence of cephalopods is the result of two interacting optical systems – the light-reflecting deeper layer of iridophores, and the light-absorbing, more superficial layer of chromatophores. In all cases, the chromatophore layer is modulatable, and in some cases, the iridophore layer also is tunable. Understanding both layers is key to understanding dynamic aquatic camouflage. We will investigate the biophysical mechanisms governing tunable reflectance using assays already developed by the Morse lab. Briefly, it previously had been shown that loliginid squid actively modulate both the intensity and wavelength of reflectance in response to the neurotransmitter, acetylcholine. Morse's group recently demonstrated that the neurotransmitter acetylcholine activates a G-protein-phospholipase-dependent signal transduction cascade that culminates in the activation of enzymatic phosphorylation of "reflectin" proteins composing the photonic Bragg stacks of the iridosome (Izumi *et al*. 2009), and that this phosphorylation changes the net charge of the reflectin molecules, driving their aggregation, with a resulting increase in refractive index concomitant with changes in the thickness and spacing of the Bragg reflectors. We will first sequence and express reflectin proteins from our two uncharacterized core species, *P. microlampas* and *O. bimaculoides*, as well as species of particular interest from our field trip to Palau and opportunistically sampled through our collaboration with MBARI. We will take advantage of next-generation "454" high-throughput DNA sequencing and transcriptional profiling technology to generate these sequences, because reflectin genes often have many copies in the genome, long stretches of unalignable sequence, and repetitive elements, which makes traditional degenerate PCR-based sequencing methods difficult. We have also developed dynamic light scattering, electron microscopic, and refractive index assays for the neurotransmitter- and phosphorylation-induced hierarchical self-assembly of the recombinant reflectin protein, and propose to use these assays with the novel reflectins we will to chart the extent to which a unified molecular and biophysical mechanism of action underlies the diversity of camouflage in cephalopods.

WORK COMPLETED

Since official fund codes were only generated at UCSB and UCSD a short time before the end of the budget period (due to the multiple processing steps at ONR, Duke and the UC system), we are currently in the very initial stages of the project and have only completed the following.

1. Four of the five PIs of our group met with the PIs of the other MURI team (led by Molly Cummings) in Austin TX. This three day meeting allowed all the PIs from both teams to present their proposed research, followed and interspersed by discussion of how to coordinate the two teams to maximize both efforts. Discussions centered in particular around field operations and it was decided that all the proposed field sites and cruises had their merits and that participation by members of both groups in several of these would be of benefit to all.

2. PIs at all three institutions have been interviewing and hiring postdocs, technicians and other personnel.

3. Johnsen at Duke (who got fund codes slightly earlier) has been purchasing capital equipment and completing work on the microspectrophotometer. This device should be completed by November 1st, and will be invaluable for measuring the visual capabilities of the model cephalopods. In addition, a prototype optomotor systems has been built and tested on a research cruise in August.

4. In September, Sweeney and Morse at UCSB hired Anna Howell, a highly qualified technician with strong expertise in animal behavior and molecular biology, to work full-time on this project. In her first three weeks on the job, Anna has been trained to perform the highly complex technique of biological transmission electron microscopy (TEM) from beginning to end, which is essential for full characterization of photonic structures. Anna has also developed a tissue sample and photography database for the project to archive separate iridescent tissue samples from individual animal organs for TEM, molecular biology and photography.

5. In June, Sweeney participated in an oceanographic research cruise in collaboration with the Monterey Bay Aquarium Research Institute, and collected tissue samples and performed pilot experiments for the MURI project. She obtained TEM-fixed samples, genetic samples and photographs, of from 12 different photonic tissues with optical structures in six species of deep-sea cephalopods. She also performed pilot optical characterization studies of these structures and tissues using a combination of reflective compound microscopy and fiber optic spectroscopy. Some of these pilot results were presented at the PI meeting in Austin in August.

6. Stramski's lab at Scripps conducted an analysis of the design of the SRA sensors with a specific purpose of reducing the size of the sensors (the outer diameter) to 2 cm. The sensors in our present Porcupine instrument have an outer diameter of about 4 cm. In this analysis, we considered various components that will be part of new SRA sensors, including custom-built photodiodes of appropriately reduced size.

RESULTS

None as of yet, for the reasons mentioned above.

IMPACT/APPLICATIONS

The systems evolved by marine animals in order to hunt, hide, and mate over hundreds of million years surpass our contemporary engineering designs for underwater vehicles. Hiding and hunting are natural tasks for our military and we believe that valuable clues will be provided by the results of our studies. The impact will hopefully affect all branches of the armed forces that have aquatic missions. This includes Special Forces, mine hunting vehicles, the submarine community, and a newest generation of underwater vehicles that could all benefit from the option of "stealth". Since visual methods play an important role in the mission profiles of all of these groups, the ability to enhance and hide from detection should be an important payoff.

RELATED PROJECTS

"Bioinspired Dynamically Tunable Polymer-Based Filters for Multi-Spectral Infrared Imaging"; DARPA; W911NF-08-1-0494; $150,000; 10-01/08-09/30/09. This work represents a "translation" of what we learned from the biomolecular mechanisms governing dynamically tunable reflectance in

cephalopods to novel routes for synthetic optical materials. Performed in collaboration with Raytheon, Inc.

"Bioinspired Dynamically Tunable Polymer-Based Filters for Multi-Spectral Infrared Imaging"; Raytheon; SB090012; $60,000; 10/01/08-09/30/09. Same project as above: This work represents a "translation" of what we learned from the biomolecular mechanisms governing dynamically tunable reflectance in cephalopods to novel routes for synthetic optical materials.

"Bioinspired Dynamically Tunable Polymer-Based Filters for Multi-Spectral Infrared Imaging"; UC Discovery Grant-Biotechnology; GCP07-10260; $39,604; 10/01/08-09/30/09. Same project as above: This work represents a "translation" of what we learned from the biomolecular mechanisms governing dynamically tunable reflectance in cephalopods to novel routes for synthetic optical materials. Performed in collaboration with Raytheon, Inc.

"Biomolecular Mechanism, Cloning, Sequencing and Analysis of Adaptive Reflecting DNAs and Proteins of Squid"; U.S. Army Research Office; W911NF-06-0285; $605,000; 07/01/06-12/31/09. Provided the molecular understanding of the mechanisms controlling dynamically tunable reflectance in cephalopods on which our present ONR project is based.